

United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol

SAM 407

Supplemental Assay Method for Titration of Marek's  
Serotype 3 (Herpesvirus of Turkeys Strain FC-126),  
Lyophilized

Date: April 4, 2005  
Number: VIRSAM0407.02  
Supersedes: PYSAM0407.01, October 29, 1998  
Standard Requirement: 9 CFR 113.330  
Contact: Scott P. Taylor, (515) 663-7598  
Karen G. Wineland, (515) 663-7662

Approvals:

/s/Donna M. Gatewood Date: 25APR05  
Donna M. Gatewood, Section Leader  
Virology Section

/s/Richard E. Hill, Jr. Date: 26APR05  
Richard E. Hill, Jr., Director  
Policy, Evaluation, and Licensing  
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde Date: 26APR05  
Rebecca L.W. Hyde, Section Leader  
Quality Assurance  
Center for Veterinary Biologics

United States Department of Agriculture  
Animal and Plant Health Inspection Service  
P. O. Box 844  
Ames, IA 50010

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may be suitable.

UNCONTROLLED COPY

Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized

Table of Contents

1. Introduction
2. Materials
  - 2.1 Equipment/instrumentation
  - 2.2 Reagents/supplies
3. Preparation for the test
  - 3.1 Personnel qualifications/training
  - 3.2 Preparation of equipment/instrumentation
  - 3.3 Preparation of reagents/control procedures
  - 3.4 Preparation of the sample
4. Performance of the test
5. Interpretation of the test results
6. Report of test results
7. Summary of revisions

Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized

## 1. Introduction

This Supplemental Assay Method (SAM) describes a procedure using chick embryo fibroblast (CEF) cell cultures, for titrating cell-free Marek's Serotype 3 (herpesvirus of turkeys Strain FC-126) used as a vaccine against Marek's disease. The vaccine is composed of lyophilized cell-free preparation of the virus and a suitable diluent.

## 2. Materials

### 2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

- 2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)
- 2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model 252)
- 2.1.3 Water-jacketed incubator with a humidified  $5 \pm 1\%$  CO<sub>2</sub> atmosphere and temperature set at  $37^{\circ} \pm 2^{\circ}\text{C}$  (Forma Scientific, Model No. 3158)
- 2.1.4 Vortex mixer (Thermolyne Maxi Mix II Model No. M37615)
- 2.1.5 Magnetic stir plate
- 2.1.6 Scissors, sterile (Roboz RS-6800)
- 2.1.7 Curved tip forceps, sterile (V. Mueller SU 2315)
- 2.1.8 Pipette (Rainin Pipetman, P1000, or equivalent)
- 2.1.9 250-ml trypsinizing flask with stir bar, sterile
- 2.2.10 Erlenmeyer flask with a stirring bar, sterile.
- 2.1.11 Hemocytometer
- 2.1.12 Bunsen burner

Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized

2.1.13 Blunt thumb forceps, sterile

**2.2 Reagents/supplies**

All reagents and supplies must be sterile.

2.2.1 Cotton swab

2.2.2 Tissue culture dish, 150 x 10-mm

2.2.3 Tissue culture dish, 100 x 20-mm

2.2.4 Plastic funnel covered with 4 layers of fine gauze

2.2.5 Polypropylene conical tube, 29 x 114-mm, sterile, 50-ml

2.2.6 Polypropylene centrifuge tubes, 250-ml

2.2.7 Roller bottles, 1000-ml (plastic)

2.2.8 Serological pipettes

2.2.9 60-mm gridded cell culture dish, tissue culture treated

2.2.10 2 dozen specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old

2.2.11 Fetal Bovine Serum (FBS)

2.2.12 L-Glutamine

2.2.13 Trypsin, 2.5%

2.2.14 Pipette tips

Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized

2.2.15 Solutions

All solutions are filter sterilized.

1. Trypsin Solution (0.25%):

Rapidly thaw frozen 2.5% (10X) trypsin in warm water. Immediately make a working solution (0.25%) by aseptically diluting the 10X solution 1:10 with Dulbecco's PBS,  $\text{CaCl}_2$  and Mg free.

2. Growth Medium:

Medium 199 (with Earles salts)(powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth (dry powder)	2.95 g
$\text{NaHCO}_3$	2.5 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
HEPES	11.97g
Fetal Bovine Serum*(gamma-irradiated)	60 ml
q.s. with distilled or deionized water	2185 ml

Adjust pH to 7.35 to 7.4 by adding  $\text{NaHCO}_3$  solution. Before use, add 1.0 ml of a 200 mM concentration of L-glutamine per 100 ml medium.

3. Maintenance Medium:

Medium 199 (with Earles salts)(powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth(dry powder)	2.95 g
$\text{NaHCO}_3$	2.75 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
HEPES	11.97 g
Fetal Bovine Serum* (gamma-irradiated)	10-20 ml
q.s. with distilled or deionized water	2142 ml

Adjust pH to 7.5 by adding  $\text{NaHCO}_3$  solution. Before use, add 1.0 ml of a 200-mM concentration of L-glutamine per 100 ml medium.

\*Previously tested for freedom from extraneous agents

**Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized**

**4. SPGA Diluent:**

Sucrose	74.62 g
KH <sub>2</sub> PO <sub>4</sub>	0.45 g
K <sub>2</sub> HPO <sub>4</sub>	1.35 g
Mono Sodium Glutamate	0.80 g
1% Bovine Albumin (Fraction V)	10.0 g
q.s. with distilled or deionized water	1000 ml

**2.2.16 Primary Chick Embryo Fibroblast (CEF) preparation**

Primary CEF cultures can be prepared from 9- to 11-day-old genetically susceptible (C/E) embryos from an ALV-negative chicken flock in the following manner, or alternate methods of preparation are acceptable:

1. Disinfect the air cell end of the egg with 70% ethanol and break open the shell with sterile forceps. Use the forceps to open the membranes, lift out the embryo, and place it in a sterile disposable 150 x 10-mm tissue culture or petri dish. Remove and discard the heads and viscera of the embryos with sterile forceps. Wash the embryo carcass several times with growth media (without FBS or L-glutamine) to remove excess blood. Place the washed embryos in a sterile dry 100 x 10-mm tissue culture or petri dish and mince them thoroughly using sharp sterile scissors.
2. To further wash, put the minced tissue into a trypsinizing flask containing 50 ml growth medium (without FBS or L-glutamine) and a magnetic stir bar. Place the flask on a stir plate and stir with a moderate vortex for 5 minutes. Allow the cells to settle and decant (and discard) the supernatant and repeat.
3. To trypsinize the tissues, first rinse the residual media from the cells by adding 10 ml of the 0.25% trypsin solution, and then immediately decant the trypsin solution. Next add 40 ml of the 0.25% trypsin solution to the flask and mix on a magnetic stir plate for 15 minutes. (Set stir plate to produce a moderate vortex.)
4. Place a sterile gauze wrapped funnel into the opening of a 250-ml conical centrifuge tube. To stop the trypsinizing action on the cells, pour 2 ml of FBS through the gauze and then pour the trypsinized

**Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized**

contents of the flask through the gauze funnel into the centrifuge tube. Bring the total volume to approximately 125 ml with growth media. Centrifuge for 10 minutes at  $250 \times g$  (1050 rpms using a Beckman J-6B centrifuge with a JS-4.2 rotor) with the temperature set at  $10^{\circ}\text{C}$ .

Resuspend the cells in growth medium to a concentration of approximately 750,000 cells per ml. Plant the cells in 60-mm plastic petri dishes (gridded or plain), 5 ml per dish (approximately  $3.75 \times 10^6$  cells). Incubate at  $37^{\circ} \pm 1^{\circ}\text{C}$  in a high-humidity atmosphere containing approximately  $5 \pm 1\%$   $\text{CO}_2$ . In 24 hours the monolayers should be complete and ready for inoculation.

### **3. Preparation for the test**

#### **3.1 Personnel qualifications/training**

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies and Quality Assurance guidelines of the Center for Veterinary Biologics (CVB) or equivalent; and training in the operation of the laboratory equipment listed in **part 2.1**.

#### **3.2 Preparation of equipment/instrumentation**

Operate all equipment/instrumentation according to manufacturers' instructions and monitor in compliance with current corresponding CVB/National Veterinary Services Laboratories (NVSL) Standard Operating Procedures (SOPs), or equivalent.

#### **3.3 Preparation of reagents/control procedures**

Prepare reference viruses in the same manner as sample preparation.

Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized

### 3.4 Preparation of the sample

#### 3.4.1 Preparation of vaccine for titration

Rehydrate the vaccine (HVT) to field strength (1 dose/0.2 ml) with the proper amount of the manufacturer's diluent. Mix thoroughly.

#### 3.4.2 Holding period

Place the vaccine bottle in an ice bath for 2 hours prior to proceeding with the titration.

## 4. Performance of the test

### 4.1 Preparing dilutions and inoculating plates

Prepare dilution blanks containing 9 ml of the manufacturer's diluent or SPGA diluent. Remove the medium from the titration plates as follows: Set the plates on a slanted surface. Remove fluid using a pipette, then allow the plates to drain an additional 60-90 seconds. Next remove the remaining fluid accumulated at the lower edge of the plates. Mix the virus suspension thoroughly by inverting several times. Put 1.0-ml of the vaccine into the 9-ml dilution blank. This makes a 1:10 dilution of the vaccine (for vaccine expected to have a relatively high-virus content, a 1:20 dilution may be used). Mix this dilution thoroughly, and inoculate 0.05 ml per plate onto the center of each of 5 plates (on a horizontal surface). Incubate the inoculated plates at  $37^{\circ}\pm 1^{\circ}\text{C}$  in a humidified atmosphere for a 30-45 minutes absorption period. Add 5 ml maintenance medium to each plate, and continue incubation at  $37^{\circ}\pm 1^{\circ}\text{C}$  in a high-humidity atmosphere of approximately  $5 \pm 1\%$   $\text{CO}_2$  for 6 days. The maintenance medium may be replaced after 2 or 3 days if the pH of the culture fluids becomes too acidic.

Titrate a known positive reference virus with each group of titrations. Uninoculated negative control cells are maintained to monitor the integrity of the cell culture system.



Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized

## 5. Interpretation of the test results

### 5.1 Controls

The titer of the positive reference must be within the established range for the test results to be valid. Uninoculated negative control cells confirm the integrity of the cell culture system.

### 5.2 Making plaque counts and calculating titer

#### 5.2.1 Counting

1. Well-developed plaques are usually observed by day 4 postinoculation. On day 6 postinoculation, make a plaque count using an inverted microscope (and a grid-adapted stage if plain plates have been used). By day 6, all primary plaques should have developed and secondary plaques should not be developed well enough to cause problems in counting using these testing methods.

2. Count all the plaques on each of the plates of the titration series. A plaque is counted as 1 regardless of size unless it has apparently arisen from 2 distinct centers. At least 4 plates should be counted for a valid test.

#### 5.2.2 Calculating plaque-forming units (PFUs)

Calculate the average number of plaques per plate and multiply this value by 40 (or 80)\*. This result will be the plaque-forming units (PFUs) per bird dose (assuming the volume of 1 bird dose is 0.2 ml).

### 5.3 Retests

Conduct retests as required by the Code of Federal Regulations, Title 9, part 9 113.8 (b) and requirements of minimum release in firm's current Outline of Production, Part V.

---

\*10 (dilution factor) times 4 (inoculum is equivalent to 1/4 bird dose) equals 40; or 20 (dilution factor) times 4 equals 80.

Supplemental Assay Method for Titration of Marek's Serotype 3  
(Herpesvirus of Turkeys Strain FC-125), Lyophilized

#### **5.4 Evaluation of test results**

**5.4.1** The 9CFR 113.8 (b) defines the criteria for a satisfactory/unsatisfactory serial.

**5.4.2** The firm's requirements of minimum release/stability titers for each Marek's vaccine are listed in the current Outline of Production, Part V, for the specific product code.

#### **6. Report of test results**

Titers are reported out as PFUs per bird dose.

#### **7. Summary of revisions**

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. No significant changes impacting the test were made from the previous protocol.

- The title change of SAM 407 is in accordance with the True Name nomenclature revision (Veterinary Biologics Notice dated June 12, 1996).
- **2.2.13** The trypsin has been changed from 0.25% to 2.5%.
- **2.2.15(1)** The Trypsin Solution has been revised for clarity.
- **2.2.15(2)** and **2.2.15(3)** HEPES has been added to the mediums and "heat-inactivated" has been changed to "gamma-irradiated" for the Fetal Bovine Serum.
- **2.2.16** The preparation of primary chick embryo fibroblasts (CEFs) has been added to the document.
- **4.1** Positive reference and uninoculated control procedures have been added for clarity.
- "Foci" or "focus" has been changed to "plaques" or "plaque" throughout the document.